

A type 2A protein phosphatase dephosphorylates the elongation factor 2 and is stimulated by the phorbol ester TPA in mouse epidermis in vivo

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Mouse epidermal cytosol contains a protein phosphatase with M_r 38000, which dephosphorylates the elongation factor 2 (EF-2) of protein biosynthesis and is stimulated after topical application of TPA to mouse skin [(1988) *Biochem. Biophys. Res. Commun.* 153, 1129–1135]. Dephosphorylation of EF-2 by this phosphatase is inhibited by okadaic acid at concentrations as low as 10^{-8} M, but not by heparin up to concentrations of 600 $\mu\text{g/ml}$. The catalytic subunit of protein phosphatase 2A (PP2A_c) with EF-2 as a substrate exhibits the same sensitivity towards okadaic acid and insensitivity towards heparin as the EF-2 phosphatase of epidermal cytosol. The catalytic subunit of protein phosphatase 1 (PP1_c) is strongly suppressed by heparin and less sensitive towards okadaic acid than PP2A_c. PP2A_c is around 50 times more efficient in dephosphorylating EF-2 than PP1_c. These data indicate that the TPA-stimulated EF-2 phosphatase in epidermal cytosol is a type 2A protein phosphatase.

Protein biosynthesis; Elongation factor 2; Protein phosphatase; Okadaic acid; Heparin

1. INTRODUCTION

Phosphorylation and dephosphorylation of the elongation factor 2 (EF-2) appears to play a major role in the regulation of protein biosynthesis. EF-2 is phosphorylated by the Ca^{2+} /calmodulin-dependent kinase III very selectively [1–4]. As yet no other substrate of this kinase has been found. Rapid and transient phosphorylation of EF-2 is observed after application of mitogens to cultured cells [5]. The role of this EF-2 phosphorylation is rather mysterious, especially since phosphorylated EF-2 was shown to be inactive in the elongation process [2,6]. Thus, dephosphorylation of EF-2 should be expected to follow the brief pulse of phosphorylation and to be essential for stimulation of protein synthesis. Indeed, we have shown recently that topical application of the tumor-promoting phorbol ester, TPA, to mouse skin stimulates the activity of an EF-2 phosphatase in epidermal cytosol [7] as well as epidermal protein synthesis [3]. This protein phosphatase or at least its catalytic subunit was found to have $M_r \approx 38000$ [7] indicating the identity with a type 1 or a type 2A phosphatase.

Here we show that the purified catalytic subunit of protein phosphatase 2A (PP2A_c) is much more efficient

in dephosphorylating EF-2 than that of protein phosphatase 1 (PP1_c). Furthermore, the TPA-stimulated EF-2 phosphatase in mouse epidermis cytosol can be inhibited by okadaic acid at concentrations as low as 10^{-8} M, but not by heparin, also indicating that the EF-2 phosphatase is a type 2A protein phosphatase.

2. MATERIALS AND METHODS

2.1. Materials

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and okadaic acid were kindly supplied by Professor Dr E. Hecker, German Cancer Research Center, Heidelberg, FRG and by Professor Dr O.-G. Issinger, University of Saarland, Homburg, FRG, respectively.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (spec. act. 3000 Ci/mmol) was from New England Nuclear (Waltham, MA, USA). Heparin was from Roth (Karlsruhe, FRG).

2.2. Methods

The keeping of animals and the following procedures were performed as described previously [3,7,8]: preparation of cytosol from mouse epidermis and from mouse pancreas, polyacrylamide gel electrophoresis and autoradiography.

2.2.1. Purification of protein phosphatases

The catalytic subunits of protein phosphatase 1 (PP1_c) and 2A (PP2A_c) were purified from rabbit muscle according to Tung et al. [9].

2.2.2. Assay for protein phosphatases

The activity of the purified enzymes was determined with ^{32}P -labelled phosphorylase *a* as a substrate. Phosphorylase *a* was phosphorylated with phosphorylase kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described previously [10]. The reaction mixture of the phosphatase assay contained 10 μl of an assay mixture (200 mM Tris-HCl, pH 7.4,

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Abbreviations: EF-2, elongation factor 2; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PP1_c, catalytic subunit of protein phosphatase 1; PP2A_c, catalytic subunit of protein phosphatase 2A

0.1% (v/v) mercaptoethanol, 1 mg/ml bovine serum albumin), 38 μ l of the enzyme preparation or buffer alone and 2 μ l [32 P]phosphorylase α . The reaction was started by the addition of the labelled phosphorylase α and, after an incubation at 30°C for 5 min, it was stopped by adding 20 μ l of trichloroacetic acid (25%, w/v) and 20 μ l of bovine serum albumin, potassium phosphate (1 mg/ml, 0.5 M, pH 7.0). After centrifugation, radioactivity of the precipitated protein as well as of an aliquot of the supernatant was counted. One unit of phosphatase activity is defined as 1 nmol phosphate liberated per min under the assay conditions.

2.2.3. Assay for the EF-2 dephosphorylating activity of epidermal cytosol

The assay was performed essentially as described previously [7]. Proteins of pancreas cytosol were phosphorylated with [32 P]ATP at 30°C for 4 min as described previously [8]. 20 μ l of phosphorylated pancreas cytosol were mixed with 30 μ l of epidermal cytosol of mice, which had received a topical application of 10 nmol TPA for 17 h. The mixture was incubated at 30°C for 15 min. In some experiments, okadaic acid or heparin was added to the incubation mixture as indicated in the text. The reaction was stopped by the addition of ice-cold 10% trichloroacetic acid. The following treatment of the samples for the separation of phosphorylated proteins by polyacrylamide gel electrophoresis was as described previously [8]. Phosphorylated EF-2, the predominant phosphorylated protein of pancreas cytosol, was made visible by autoradiography of the gels and could be quantitated by scanning of the autoradiograms or by cutting out and counting the piece of the gel containing EF-2.

2.2.4. Assay of the EF-2 dephosphorylating activity of PP1_c and PP2A_c

20 μ l of 32 P-labelled pancreas cytosol were incubated at 30°C for 15 min with 20 μ l of Tris/EDTA buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1 μ l of purified PP1_c or PP2A_c (final concentrations are given in the text). In some experiments, 1 μ l of okadaic acid

in acetone or 1 μ l of heparin in Tris/EDTA buffer at various concentrations, as indicated in the text, was added to the incubation mixture. The reaction was stopped by addition of 10% trichloroacetic acid and pelleted proteins were processed for polyacrylamide gel electrophoreses as described previously [8].

3. RESULTS

Mouse pancreas cytosol was used to prepare [32 P]EF-2, as previously described [7]. Cytosol proteins were phosphorylated with [32 P]ATP, using the endogenous Ca^{2+} /calmodulin-dependent kinase III. Under these conditions EF-2 is one of the most prominent phosphorylated proteins in pancreas cytosol (see figs 1b, 2 and [7]). The phosphorylated cytosol proteins were then incubated at 30°C for 15 min with buffer alone, with the catalytic subunit of protein phosphatase 1 (PP1_c) or with the catalytic subunit of protein phosphatase 2A (PP2A_c) at various concentrations. As shown in fig. 1a and b, PP2A_c exhibited strong EF-2-dephosphorylating activity at a concentration around 0.8 U/ml. PP1_c was unable to dephosphorylate EF-2 even at a 2 times higher concentration (1.8 U/ml) and at 5.5 U/ml it caused not more than 25% dephosphorylation of EF-2 (fig. 1a). It can be estimated that in order to obtain 50% 32 P-release from phosphorylated EF-2, around 50 times more PP1_c than PP2A_c would be required. At the concentrations used, PP2A_c did not release any 32 P from other cytosol proteins than EF-2 (fig. 1b). When we used phosphorylated

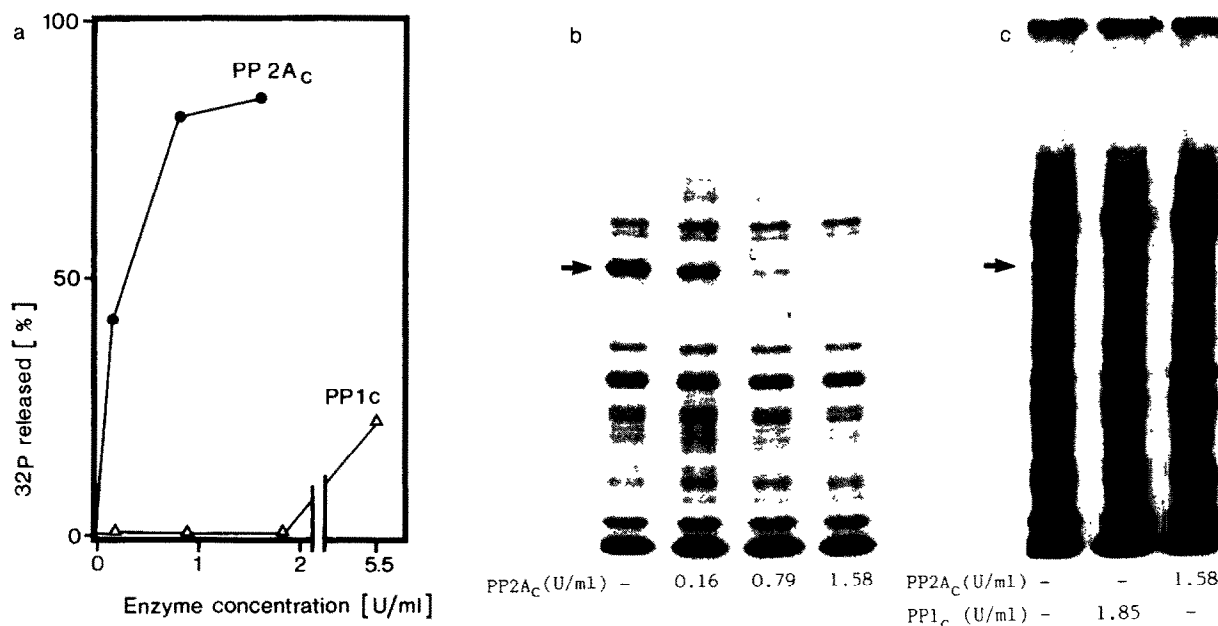


Fig.1. Dephosphorylation of phosphorylated EF-2 with PP1_c and PP2A_c. One unit of phosphatase activity is defined as 1 nmol phosphate liberated per minute under the assay conditions (see section 2). (a) Phosphorylated proteins of mouse pancreas cytosol containing EF-2 were incubated at 30°C for 15 min with buffer alone, with purified PP1_c or PP2A_c at the indicated concentrations and separated by gel electrophoresis. For the experimental details see section 2. The EF-2 bands were cut out of the gel and the radioactivity measured. 32 P-release from EF-2 is given in percent (incubation with buffer alone = 0%). Each value represents the mean of 2 experiments. (●—●) PP2A_c; (△—△) PP1_c. (b) Phosphorylated cytosol proteins were incubated with various concentrations of PP2A_c as in (a), separated by gel electrophoresis and made visible by autoradiography (see section 2; EF-2 is indicated by an arrow). (c) Phosphorylated proteins of mouse spleen cytosol were incubated with buffer alone, with 1.58 U/ml of PP2A_c or 1.85 U/ml of PP1_c and processed as in (b). EF-2 is indicated by an arrow.

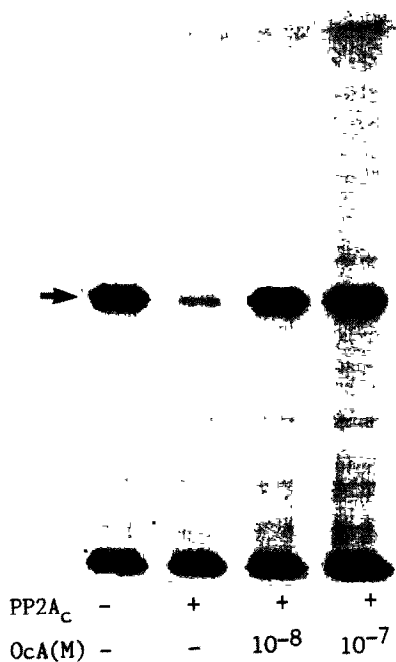


Fig. 2. Inhibition of PP2A_c-catalyzed EF-2 dephosphorylation by okadaic acid. Phosphorylated proteins of pancreas cytosol were incubated with 1.58 U/ml of PP2A_c as in fig. 1a. 1 μ l okadaic acid dissolved in acetone was added at the indicated final concentrations simultaneously with PP2A_c. 1 μ l acetone was added to the control incubation with PP2A_c alone. Precipitated proteins were separated by gel electrophoresis and made visible by autoradiography (see section 2; EF-2 is indicated by an arrow).

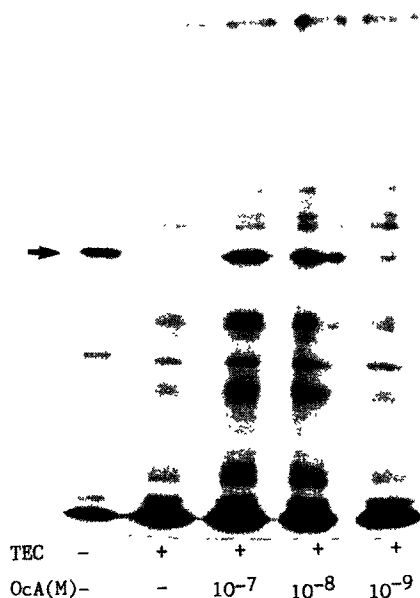


Fig. 3. Inhibition of EF-2 phosphatase by okadaic acid in epidermal cytosol of TPA-treated mice. TPA (10 nmol) was applied topically to mouse skin and after 17 h epidermal cytosol was prepared. Pancreas cytosol containing phosphorylated EF-2 was mixed with the epidermal cytosol (TEC) and incubated at 30°C for 15 min with and without okadaic acid (OcA) at the indicated concentrations. After precipitation by 10% trichloroacetic acid, the phosphorylated proteins were separated by gel electrophoresis. For the details of the procedure see section 2 and [7,8]. EF-2 is indicated by an arrow.



Fig. 4. Inability of heparin to suppress PP2A_c-catalyzed EF-2 dephosphorylation as well as the EF-2 phosphatase in epidermal cytosol of TPA-treated mice. The experiments were performed as described in figs 2 and 3, respectively, except that 50 μ g/ml heparin (Hep) in Tris/EDTA buffer instead of okadaic acid was added to the incubation mixture. Therefore, just the EF-2 band of the autoradiogram is shown.

cytosol proteins from mouse spleen, again PP2A_c (1.58 U/ml) released ³²P just from EF-2 and PP1_c (1.85 U/ml) was totally inactive (fig. 1c).

PP2A_c-catalyzed EF-2 dephosphorylation in mouse pancreas cytosol could be inhibited completely by 10⁻⁷ and 10⁻⁸ M okadaic acid, by adding okadaic acid simultaneously with PP2A_c to the cell-free system (fig. 2).

As reported previously [7], topical application of the phorbol ester TPA to mouse skin strongly increased the activity of an EF-2 phosphatase in epidermal cytosol. After incubation of phosphorylated cytosol proteins of mouse pancreas with epidermal cytosol of TPA-treated mice, ³²P was released from phosphorylated EF-2 (see [7] and fig. 3). Addition of okadaic acid to this incubation mixture at the final concentration of 10⁻⁷ and 10⁻⁸ M suppressed the ³²P-release from EF-2 completely. With 10⁻⁹ M okadaic acid still weak inhibition of EF-2 dephosphorylation was observed (fig. 3). Heparin (50 μ g/ml) neither inhibited EF-2 dephosphorylation by the purified PP2A_c nor EF-2 dephosphorylation by epidermal cytosol of TPA-treated mice (fig. 4). Heparin (up to 600 μ g/ml) was ineffective in this respect (data not shown).

4. DISCUSSION

Activation of EF-2 by its dephosphorylation might be one crucial process in the regulation of protein biosynthesis [2,6,7]. Therefore, the nature of the respective protein phosphatase is of great interest. Topical application of the phorbol ester TPA to mouse skin induces an increase in the activity of an EF-2 phosphatase in epidermal cytosol [7]. This protein phosphatase has *M_r* 38000 which would be in accordance with the molecular mass of the catalytic subunit of a type 1 or type 2A phosphatase [7]. The purified catalytic subunit of type 2A phosphatases (PP2A_c) is around 50 times more active in the dephosphorylation of EF-2 than that of type 1 phosphatases (PP1_c). This shows, at least in vitro, a preference of phosphorylated EF-2 for PP2A_c and can be taken as a first indication for the TPA-stimulated EF-2 phosphatase being a type

2A rather than a type 1 phosphatase. Furthermore, at the low concentrations used, PP2A_c appears to be highly specific for EF-2, since no other cytosol proteins are dephosphorylated.

Recently, okadaic acid was reported to inhibit various types of protein phosphatases to a different extent [11]. According to this report, 10⁻⁸ M okadaic acid caused more than 80% suppression of PP2A_c activity, but almost no suppression of PP1_c activity with phosphorylase *a* or myosin light chain as substrates. In our hands, PP2A_c shows the same high sensitivity towards okadaic acid with EF-2 as a substrate. The TPA-stimulated EF-2 phosphatase in epidermal cytosol can be inhibited completely by 10⁻⁸ M okadaic acid, indicating the identity of its catalytic subunit with PP2A_c.

Heparin is known to be a strong inhibitor of PP1 (*K_i* = 8 µg/ml [12,13]). According to Gergely et al. [12], heparin up to 50 µg/ml does not suppress PP2 activity. We tested heparin at concentrations up to 600 µg/ml and did not observe any inhibition of PP2A_c activity with EF-2 as a substrate. The TPA-stimulated EF-2 phosphatase in epidermal cytosol is also not inhibited by heparin at concentrations up to 600 µg/ml. Thus, in accordance with the other results, we can exclude that it is a type 1 phosphatase.

Taken the results together, the high EF-2 dephosphorylating activity of PP2A_c in vitro, the molecular weight of the TPA-stimulated EF-2 phosphatase [7], its sensitivity towards okadaic acid and its insensitivity towards heparin, we can conclude that most likely TPA stimulates a type 2A protein

phosphatase in mouse epidermis, which plays a major role in the dephosphorylation of EF-2 in vivo and thus in the regulation of protein synthesis. The mechanism by which TPA stimulates the EF-2 phosphatase of the 2A type is presently under investigation.

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